REVIEW

by Dr. Vesela Tsvetanova Tsakova-Stancheva

Professor, Institute of Physical Chemistry, Bulgarian Academy of Sciences

on the Ph.D. thesis for awarding the educational and scientific degree *Doctor*
Field of Higher education: 4. Natural Sciences, Mathematics and Informatics,
Professional direction 4.2. Chemical Sciences
Doctoral Program *Physical Chemistry*

Author: Mariya Genova Pimpilova
Subject: Modification of glassy carbon electrodes with electrodeposited gold or 2d-nanomaterials: characterization and applications
Scientific supervisor: Assoc. Prof. Dr. Nina Dimitrova Dimcheva - Plovdiv University *Paisii Hilendarski*

1. General description of the submitted materials

By Order No. RD-21-245 of 30.01.2024 of the Rector of the University of Plovdiv *Paisii Hilendarsk*i (PU) I have been appointed as a member of the scientific jury for the procedure for the defense of a PhD thesis on *Modification of glassy carbon electrodes with electrodeposited gold or 2D-nanomaterials: characterization and applications* for the acquisition of the educational and scientific degree *Doctor* in the field of higher education 4. Physical Chemistry. The author of the dissertation is Maria Genova Pimpilova – Ph.D. student at the Department of Physical Chemistry with scientific supervisor Assoc. Prof. Dr. Nina Dimitrova Dimcheva from PU.

The set of materials submitted by Maria Pimpilova is in accordance with Article 36 (1) of the Regulations for the Development of the Academic Staff of PU and includes the following documents:

- Application to the Rector of PU for the disclosure of the procedure for dissertation defence;
- CV in European format;
- protocol of preliminary discussion in the Department of Physical Chemistry;
- dissertation;
- summary in Bulgarian and English;
- list of scientific publications on the subject of the dissertation;
- copies of the publications on the dissertation topic;

- declaration of originality and authenticity of the attached documents;
- statement of compliance with the minimum requirements

The PhD student has attached two scientific publications in full text. A list of citations to the PhD student's work is included in the dissertation (p. 132).

2. Brief biographical data about the PhD student

Maria Pimpilova graduated from the BSc Chemistry programme at PU in 2017 and completed her MSc in Food Chemistry at the same university in 2018. From January 2020 to December 2023, she was a researcher at the Center of Technology at PU conducting research in the field of Biosensors. From March 2020 to the present, she holds a position as Assistant Professor in the Department of Physical Chemistry of the University. In the period 2019-2024, she was involved in the implementation of two contracts funded by the Bulgarian National Science Fund and a contract for the Centre of Competence *Personalized Innovative Medicine - PERIMED*, funded by OP NOIR. Since 2022, she has been implementing a project under the *Young Scientists and Postdoctoral Fellows Programme - 2*, funded by the Ministry of Education and Science. This project is in the field of *Electrocatalytic Properties of Modified Electrode Materials* and is directly related to the thesis topic.

3. Relevance of the subject matter and appropriateness of the set goals and objectives

The topic of the thesis falls within the field of physical chemistry and in particular the use of electroanalytical methods for the detection of biologically relevant substances. The thesis is devoted to the preparation, characterization and use of specifically modified electrode materials as electrochemical sensors for catecholamines, dopamine and L-epinephrine, and for hydrogen peroxide and its organic homologues. The topic of this dissertation is relevant and related to the search for suitable modified electrodes providing rapid, reliable and cost-effective determination of substances involved in biochemical processes in the human body.

In the literature there is a considerable number of publications devoted to the electrochemical detection of catecholamines and hydrogen peroxide. Nonetheless, research continues on different types of electrode materials that could improve the basic characteristics of electrochemical sensing materials such as sensitivity, detection limit, concentration interval of linear electroanalytical response, stability, durability, etc. The thesis has two main tasks:

- preparation of enzyme electrode by electrodeposition of gold on glassy carbon electrode and subsequent covalent immobilization of laccase enzymes for the electrochemical detection of catecholamines;
- modification of glassy carbon electrodes with graphitic carbon nitride (g-C_3N_4), pure or doped with metal oxides, suitable for the determination of peroxide compounds (hydrogen peroxide and tertiary butyl hydroperoxide)

Attempts to use laccase-based enzyme electrodes for the determination of catecholamines began in the 1990s with direct immobilization of laccase on a glassy carbon electrode. Subsequently, electrodes made of carbon paste, carbon nanotubes, platinum or gold have been used to immobilize the enzyme. Electrodes prepared by pre-synthesized gold nanoparticles mixed with carbon paste and laccase were also investigated. In this thesis, a different approach was chosen to combine gold and laccase in order to obtain a larger electroactive surface area of the catalytic metal and a stable covalent coupling of the enzyme to the metal phase.

Nitrogen-containing carbons, in particular g-C_3N_4, have been the subject of considerable attention due to the role of nitrogen as a strong electron donor, which stabilizes π-bonds in the structure and leads to improved stability, electron transfer rate, and hence durability of the carbon carriers when used for electrocatalytic purposes. Along with the research dedicated to pure g-C_3N_4, the possibility of its further modification with metal oxides is being intensively studied with a view to various photoelectrochemical and sensing applications. Among the metal oxides used in combination with g-C_3N_4 for electrochemical applications (photoelectrocatalysis, supercapacitors, etc.), TiO_2, ZnO, and WO_3 have been most frequently studied. The combination of g-C_3N_4, and metal oxides such as MgO, Bi_2O_3 and Co_3O_4 (the subject of this thesis) and their application for electroanalytical purposes is rarely encountered in the literature, and in the case of hydrogen peroxide determination, has not been studied until now. In this sense, the problem developed in this part of the dissertation is relevant and meets the current searches aiming, on the one hand, to avoid the use of noble metals, and on the other hand, to improve the operating environment of catalytic components with low electrical conductivity (such as Co_3O_4) when used for electroanalytical purposes.

4. Knowledge of the problem

The literature review included in this dissertation occupies 32 of the 131 pages of the dissertation and refers to 218 publications in the international literature. Essentially, the review

attempts to cover three broad topic areas: (a) preparation of modified electrodes; (b) applications of modified electrodes for electroanalytical purposes; (c) electrochemical methods for electrode characterization and for electroanalytical studies.

The preparation of modified electrodes has been the subject of thousands of publications in the scientific literature, therefore covering such a broad field of research is an ambitious task. The main focus of the Ph.D. student is the modification of carbon materials (glassy carbon, graphene, graphene oxide, carbon nitride, etc.) with metallic micro- and nanostructures and transition metal oxides. These are also the systems relevant to the research presented in this thesis. As far as the electroanalytical applications of the modified electrode materials are concerned, emphasis is placed on the electroanalytical determination of hydrogen peroxide and of catecholamines. In the section devoted to catecholamines, the review is limited to biosensors (i.e. enzyme-modified electrode) with particular attention paid to the laccase enzyme used in the thesis. The last part of the literature review, dedicated to electrochemical methods, briefly presents all electrochemical measurement methods used in the thesis.

The literature review serves as a basis in defining the clearly stated aim of the thesis, namely the development of modified electrodes as electrochemical sensors for two types of biologically relevant compounds, the catecholamines, dopamine and L-epinephrine, and hydrogen peroxide and its organic homologues. There is no doubt that the Ph.D. student knows the state of the art and presents a considerable amount of literature that serve to bring out the separate tasks of the dissertation.

5. Research methodology

In the Experimental Part (third chapter of the thesis), the various preparative techniques required for the preparation of the modified electrodes and the suitably selected electrochemical measurement methods necessary for the characterization of the elctrode materials and for electroanalytical measurements are described. The description of all the techniques used is detailed and enables one to follow all the experimental approaches for the preparation of catalytically active glassy carbon electrodes by different types of modification: electrodeposition of gold; drop casting of suspensions containing graphitic carbon nitride (g-C_3N_4), pure or doped with metal oxides such as MgO, Bi_2O_3, Co_3O_4; immobilization of the laccase enzyme on gold-modified glassy carbon electrodes; immobilization of the catalase enzyme on glass substrates. Cyclic voltammetry and electrochemical impedance spectroscopy, complemented with optical or electron microscopy, were used for electrochemical

characterization of the modified electrodes in background electrolyte and in the presence of the studied substances. Electroanalytical measurements were carried out by the methods of differential pulse voltammetry and chronoamperometry. The order of the electrochemical reaction in the case of hydrogen peroxide reduction using a Co- g-C_3N_4 modified electrode was determined using a rotating disk electrode. The combination of a variety of electrochemical methods used allows to achieve the objectives of the study and corresponds to modern approaches in electrochemical experiment.

6. Characteristics and evaluation of the thesis

The content of the dissertation is presented in five chapters: 1.Literature review, 2.Research setting, 3.Experimental part, 4.Results and discussion and 5. 2.

The main body of the thesis (52 pages in Chapter 4) is structured into three main themes: 1. Glassy carbon electrode modified with electrochemically deposited gold - preparation, characterization and applications; 2 Glassy carbon electrode modified with two-dimensional nanomaterials based on graphitic carbon nitride (g-C_3N_4); 3. Electrocatalytic activity of modified Co-g-C_3N_4/Nafion^{TM} electrode in non-aqueous medium.

The first part of the experimental studies is devoted to the preparation of a biosensor for catecholamines using two alternative methods for gold electrodeposition and additional optimization performed with respect to the modifier (cysteine or cystamine) used for enzyme immobilization. It is found that electroanalytical determination of the two catecholamines (dopamine and L-epinephrine) was possible at detection limits of 0.037 μM for dopamine and 0.054 μM for L-epinephrine. A linear concentration dependence of the electroanalytical signal is established for concentration up to 120 μM and 190 μM, respectively. The applicability of the developed biosensor is demonstrated by measuring the concentration of the two catecholamines in injection solutions.

The part related to the modification of glassy carbon electrodes with pure graphitic carbon nitride (g-C_3N_4) and with additionally incorporated metal oxides (MgO, Bi_2O_3 and Co_3O_4) presents the significant efforts to optimize the electrode coatings with respect to several factors: type of metal oxide, ratio of nitride to metal oxide phase, type (Nafion^{TM} or glutar aldehyde) and amount of the binding agent, pH of buffer solution, as well as electroanalytical measurement parameters. A significant amount of experimental work is performed to select a modified (Co- g-C_3N_4)/Nafion^{TM} type electrode that is suitable for electrochemical detection of hydrogen peroxide and tertiary butylhydroperoxide over a wide concentration range in aqueous

media. The catalytic electrode thus optimized is used to electrochemically monitor the activity of the enzyme catalase immobilized on a glass substrate. For this purpose, the difference in electrocatalytic currents measured in the absence and presence of the immobilized catalase was interpreted with the Michaelis-Menten equation. This new electrochemical approach for the study of enzyme activity is without analogue in the scientific literature.

The third part of the presentation of the original results is devoted to the use of the developed peroxide catalytic electrode for the determination of water-insoluble organic peroxide compounds (benzoyl peroxide as model peroxide and oxidized anti-cellulite massage oil as real sample). The results in this part reveal the feasibility of real application of the developed peroxide catalytic electrode for electroanalytical determination of peroxide numbers of vegetable oils.

All the experimental studies are presented with sufficient detail to follow the results and the reliability of the conclusions drawn. The conclusions are correctly formulated on the basis of the main results obtained in the thesis. Very good impression is made by the fact that in each part there is a critical analysis of the stability of the obtained modified electrodes and their suitability for long-term use.

7. Contributions and Significance of the Development for Science and Practice

The contributions of the Ph.D. thesis relate generally to the preparation and systematic characterisation of modified electrodes to find application as electrochemical sensors for the determination of biologically relevant substances involved in human metabolism or those present in products of the cosmetic or food industry. These can be summarised as follows:

(a) A bioenzyme sensor for the catecholamines dopamine and L-epinephrine based on electrodeposited gold and immobilized laccase has been developed that exhibits lower detection limits and wider concentration ranges of linear electroanalytical signal compared to previously developed electrodes using pre-synthesized gold nanoparticles and immobilized laccase.

(b) A modified electrode with high electrocatalytic activity based on the synergic effect of the catalyst Co-g-C_3N_4 and the ionomer Nafion^{TM} has been developed that allows the determination of water-soluble peroxides (hydrogen peroxide and tertiary butylhydroperoxide) over a wide concentration range from 0.4 to 14 mM.

(c) A pioneering electrochemical method has been developed for the determination of the activity of the immobilized enzyme catalase, which is without analogue in the scientific literature.

(d) Based on a Co-g-C_3N_4 and Nafion^{TM} type catalytic electrode, an electrochemical method for the determination of peroxide number of vegetable oils was developed as an alternative to the widely used titrimetric methods.

Overall, I highly appreciate the contributions of this dissertation, which are of a scientific and applied nature and are related to opportunities for implementation in practice.

8. Assessment of the publications on the dissertation

Maria Pimpilova's dissertation is based on two papers in English, published in 2022 in refereed international journals, *Biosensors* and *Catalysts*. Both journals are among the most prestigious in their respective specific scientific fields and rank as follows: *Biosensors* in the first quarter (Q1) in the order of journals by impact factor in the field of *Analytical Chemistry* and *Catalysts* in the second quarter (Q2) in the order of journals by impact factor in the field of *Physical Chemistry*.

The publications submitted by Ph.D. student Pimpilova are the result of a collective work, and besides the Ph.D. student and her supervisor, two researchers from PU (in the publication on hydrogen peroxide reduction) and one from the Technical University of Sofia - Plovdiv branch (in the publication on dopamine oxidation) participate as co-authors. As it is clear from the text of the dissertation, the co-authors from the Faculty of Chemistry of PU (Stoyanova and Kolcheva) have synthesized, characterized and provided the catalysts subsequently used by the Ph.D. student for modification of glassy carbon electrodes and electrochemical studies of hydrogen peroxide reduction. In the two publications included in the thesis, the Ph.D. student is listed first in the order of co-authors, which makes me think that she was a major contributor in conducting all of the electrochemical measurements and obtaining the main experimental results presented in the thesis.

The PhD student is also co-author of a patent application (№ BG|P|2023|113803), registered in the Patent Office of the Republic of Bulgaria on 27.10.2023 with the title *Electrochemical method for the quantification of peroxide compounds* and authors V. Kolcheva, N. V. Stoyanova, M. Pimpilova.

The list of citations found for the PhD student's work shows that the article published in *Biosensors* has six citations (without self-citations) and the one published in *Catalysts* has two

citations (without self-citations). These citations by foreign authors were obtained in less than two years after the publications appeared, which is a clear indication of the relevance of the topic and the significance of the results obtained.

9. Personal participation of the PhD student

As noted above, the doctoral student is first author on the two publications on which the dissertation is based. This leads me to believe that the experimental papers presented are the personal work of the Ph.D. student. It is also noteworthy that she commented competently on questions posed to her during the preliminary discussion of the dissertation, demonstrating an excellent knowledge of the details of the experiment and of the scientific problem as a whole. Maria Pimpilova has presented her scientific results at six international and national conferences and seminars, indicating her active role in presenting the research included in the thesis.

Although I can only give an estimate of the PhD student's personal involvement based on circumstantial evidence, I believe that it has been decisive for the considerable amount of experimental work carried out on the modification of electrodes with different types of modifiers, and optimization with respect to the final electroanalytical objectives.

10. Summary

The Summary of the thesis, presented in Bulgarian and in English, consists of 32 pages, which include 27 figures and three tables. In terms of length, it meets the requirement of the Regulations for the Development of the Academic Staff of PU. The Summary has been carefully prepared and reflects the main results achieved in the thesis, while at the same time allowing to follow the overall conduct of the experimental studies and the conclusions drawn.

11. Critical remarks and recommendations

I have no substantive critical comments, but rather some general observations and remarks in relation to clarifying details of the presentation:

It seems to me that the literature review would have benefited from a narrower focus on electrochemical and bioelectrochemical sensor electrodes for the determination of hydrogen peroxide and catecholamines. A more focused overview of the literature devoted to sensors for dopamine and L-epinephrine based on laccase-modified and gold-modified electrodes would have provided an opportunity to comment on the detection mechanisms of the analytes studied and would have clearly distinguished the Ph.D. student's research in this area. Regarding the

second task, a focus on the g-C_3N_4 and metal oxide nanocomposites developed so far and their electrochemical and sensing applications would serve to better highlight the pioneering contributions of this thesis.

Notes regarding clarification of details of the thesis:

(a) the equivalent schemes (Fig. 3 B) used to interpret the electrochemical impedance data are different for the "pure" glassy carbon electrode and the one, modified with gold; the Warburg impedance, although commented in the text, is not included in the equivalent scheme corresponding to the glassy carbon electrode;

(b) with a view to understanding the role of laccase, it would be useful to specify the chemical forms of the oxidized and reduced forms of dopamine and L-epinephrine, in the mechanism shown on page 67 of the thesis;

(c) catecholamine oxidation studies were performed at a constant pH value (pH 4) without mention of what determines work under these conditions;

(d) the difference in catalytic currents measured in the absence and presence of catalase (Fig. 37) was interpreted in a narrow concentration range (up to about 4.5 mM hydrogen peroxide), although the experiment was conducted at concentrations up to 10 mM (Fig. 36).

Finally, there are some purely technical omissions, e.g., the units for current and concentration in the regression equation (p. 87) are probably [A] and [mol], respectively, rather than [nA] and [mmol], the regression equation corresponding to the data in Fig. 28 is not mentioned in the text, etc.

These remarks do not diminish the significance of the studies presented and their original contributions.

12. Personal impressions

I have no personal impressions of the PhD student.

13. Recommendations for future use of the dissertation contributions and results

I think that the modified electrodes developed in this dissertation open the prospect of exploring their applicability as electrochemical sensors for other substances. For example, the electrode modified with Co-g-C_3N_4 could be suitable for the determination of dopamine or phenolic compounds such as bisphenol A, octylphenol, etc. The pioneering approach for the

electrochemical determination of enzyme activity, probed in the case of immobilized catalase, could probably be optimized in terms of enzyme immobilization or applied in catalase solution. This unconventional approach could also be useful for investigating the activity of other enzymes.

After evaluation of the patent application submitted, it is likely that further implementation of the electrochemical method for peroxide number assessment will become possible in various products of the food and cosmetic industries for which the quality of the vegetable oils used is essential.

CONCLUSION

The Ph.D. thesis contains scientific and applied results that represent an original contribution to science and meet all the requirements of the Law for the Development of Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the Implementation of the LDASRB and the relevant Regulations of the Plovdiv University *Paisii Hilendarski*.

The presented dissertation shows that the Ph.D. student Maria Pimpilova possesses in-depth knowledge and experimental skills in the scientific specialty *Physical Chemistry*, demonstrating qualities and skills for independent scientific research.

Because of the above, I confidently give my positive assessment of the research conducted, presented by the above-reviewed Dissertation, Summary, Results and Contributions, and I propose the honorable scientific jury to award the educational and scientific and degree *Doctor* to Maria Genova Pimpilova in the Field of Higher education: 4. Natural Sciences, Mathematics and Informatics, Professional direction 4.2. Chemical Sciences, Doctoral Program *Physical Chemistry.*

29.03.2024 Reviewer:

Prof. DSc Vesssela Tsakova-Stancheva